FEBS 28342 FEBS Letters 565 (2004) 163–166

The effect of phalloidin and jasplakinolide on the flexibility and thermal stability of actin filaments

Balázs Visegrády[a], Dénes Lőrinczy[a], Gábor Hild[b], Béla Somogyi[a,b], Miklós Nyitrai[a,*]

[a] Department of Biophysics, Faculty of Medicine, University of Pécs, Szigeti str. 12, Pécs H-7624, Hungary
[b] Research Group for Fluorescence Spectroscopy, Office for Academy Research Groups, Hungary

Received 16 January 2004; revised 25 March 2004; accepted 30 March 2004

First published online 15 April 2004

Edited by Michael R. Bubb

Abstract In this work the effect of phalloidin and jasplakinolide on the dynamic properties and thermal stability of actin filaments was studied. Temperature dependent fluorescence resonance energy transfer measurements showed that filaments of Ca-actin became more rigid in the presence of phalloidin or jasplakinolide. Differential scanning calorimetric data implied that the stiffer filaments also had greater thermal stability in the presence of phalloidin or jasplakinolide. The fluorescence and calorimetric measurements provided evidences that the extent of stabilization by jasplakinolide was greater than that by phalloidin.

Keywords: Actin; Jasplakinolide; Phalloidin; Fluorescence resonance energy transfer; Differential scanning calorimetry

1. Introduction

Phalloidin, a cyclic peptide from *Amanita phalloides*, can tightly bind to actin filaments and stabilizes their structure [1,2]. Phalloidin stabilized actin filaments were extensively used in in vitro studies. Fluorescent derivatives of phalloidin were also applied to visualize the architecture of the actin cytoskeleton by fluorescence microscopic methods in intracellular studies. The effect of another cyclic peptide, jasplakinolide, from a marine sponge (*Jaspis johnstoni*) on actin filaments is understood to a much lesser extent than that of phalloidin. It is known that jasplakinolide binds to F-actin competitively with phalloidin [3]. Jasplakinolide accelerates actin polymerization [3], promotes actin polymerization under non-polymerizing conditions and lowers the critical concentration of actin assembly in vitro [4]. Although phalloidin can stabilize actin oligomers, similar effect from jasplakinolide was not observed [5]. Another important difference between the two drugs is that in contrast to phalloidin, jasplakinolide readily enters mammalian cells [5]. It appears that the effect of jasplakinolide is similar to that of phalloidin in some aspects, while other effects are different for the two toxins.

In this study, we compared the effect of phalloidin and jasplakinolide on the dynamic properties and thermal stability of actin filaments. The fluorescence resonance energy transfer (FRET) experiments showed that both phalloidin and jasplakinolide made the filaments of Ca-actin more rigid. The differential scanning calorimetric results were in accordance with the fluorescence data indicating that the stiffer filaments in the presence of phalloidin or jasplakinolide had greater thermal stability. Despite the similarities between the effect of phalloidin and jasplakinolide, both the fluorescence and calorimetric measurements provided evidences that the extent of stabilization by jasplakinolide was greater than that by phalloidin.

2. Materials and methods

2.1. Chemicals

KCl, $MgCl_2$, $CaCl_2$, NaOH, Mops, Tris, *N*-(((iodoacetyl)amino)ethyl)-5-naphthylamine-1-sulfonate (IAEDANS), dimethylformamide and phalloidin were obtained from Sigma Chem. Co. (St. Louis, MO, USA). 5-(Iodoacetamido)fluorescein (IAF), fluorescein cadaverine (FC) and jasplakinolide were purchased from Molecular Probes (Eugene, OR, USA). ATP and 2-mercaptoethanol were obtained from MERCK (Darmstadt, Germany) and NaN_3 was purchased from Fluka (Switzerland).

2.2. Protein preparation and fluorescent labeling of the samples

Acetone-dried powder of rabbit skeletal muscle was obtained as described previously [6] and actin was prepared according to the method of Spudich and Watt [7]. Actin was labeled with fluorescent probes (IAEDANS (on Cys^{374}), IAF (Cys^{374}) or FC (Gln^{41})) as described previously [8–10]. The labeling ratios (the ratio of the probe concentration to the actin concentration) for IAEDANS, IAF and FC were 0.9, 0.6 and 0.6, respectively. For one set of FRET experiments, aiming to characterize the intramonomer dynamics of protomers, actin was labeled with IAEDANS and FC and the double labeled actin monomers were mixed with unlabeled actin in a 1:10 ratio before polymerization. When the contribution of unlabeled actin was increased in control experiments (up to 1:15) the measured FRET efficiencies were identical to those measured with the ratio of 1:10, indicating that the effect of intermonomer FRET was negligible under these conditions. Preparing the samples this way, therefore, assured that each donor could only interact with one acceptor located on the same actin monomer. For another set of FRET experiments, where the intermonomer dynamics was studied, actin monomers were labeled with either IAEDANS or IAF separately, and the single labeled monomers were mixed in a 1:9 ratio before polymerization. In these experiments unlabeled actin was not added to the samples. The total concentration of the actin filaments during the experiments was 20 μM. To study the effect of toxins, the samples were incubated with phalloidin or jasplakinolide in a 1:1 molar ratio at 4 °C for 12 h.

2.3. Steady-state fluorescence experiments

The steady-state fluorescence measurements were performed with a Perkin–Elmer LS50B Luminescence Spectrometer equipped with a thermostatic sample holder flushed with dry air against the water

* Corresponding author. Fax: +36-72-536261.
E-mail address: miklos.nyitrai@aok.pte.hu (M. Nyitrai).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.03.096

condensation. The experiments were carried out at pH 8.0 in a buffer containing 2 mM Tris/HCl, 0.2 mM ATP, 0.005% NaN_3, 0.5 mM 2-mercaptoethanol, 100 mM KCl and 2 mM $CaCl_2$ (buffer A). The excitation wavelength for IAEDANS was 350 nm. The optical slits were set to 5 nm in both the excitation and emission side. In FRET experiments the fluorescence emission of the donor (IAEDANS) was recorded between 400 and 460 nm, and the area under the spectral curve was used to calculate the FRET parameters. The fluorescence spectra were corrected for the inner filter effect with the absorption spectra of the same sample by using the following equation:

$$F_{corr} = F_{obs}\text{antilog}(A_{ex} + A_{em}) \quad (1)$$

where F_{corr} and F_{obs} are the corrected and measured fluorescence intensities, respectively, and A_{ex} and A_{em} are the absorption of the sample at the excitation and the emission wavelengths.

2.4. Theoretical considerations

The efficiency of FRET (E) can be determined from the donor intensities by using the following equation:

$$E = 1 - (F_{DA}/F_D) \quad (2)$$

where F_{DA} and F_D are the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively.

The normalized FRET efficiency, f', was defined as the ratio of the FRET efficiency to the fluorescence intensity of the donor measured in the presence of the acceptor [11]:

$$f' = E/F_{DA} \quad (3)$$

An experimental strategy has been developed to obtain information regarding the flexibility of the different forms of a protein by temperature dependent FRET measurements [11]. The temperature profile of f' (Eq. (3)) is characteristic for the flexibility of the protein matrix between the applied donor and acceptor. When these FRET results are interpreted in terms of flexibility, it is assumed that the changes of the temperature alter the relative fluctuation of the fluorophores around an equilibrium donor–acceptor position and the donor–acceptor distance distribution becomes wider with increasing temperature. The increase of the amplitude of the fluctuations is expected to increase uniformly the value of f' without causing any definite break on the slope of its temperature profile [11]. The method is sensitive to different kinds of protein motions, which can alter the donor–acceptor distance distribution.

It should be emphasized that the method cannot provide the measure of absolute flexibility. Instead, the term flexibility is related to how easily the donor–acceptor distance distribution widens as a response to the additional energy represented by the increase in temperature. Comparison of the temperature-induced changes therefore provides information regarding the differences of flexibility between the different forms of a protein. The steeper temperature profile of the f' corresponds to the more rigid form of the protein. However, another molecular mechanism can also be responsible for the temperature dependence of f', which results in a temperature induced change of the equilibrium distance between the donor and acceptor reflecting conformational transition within the protein matrix. In such a case the temperature profile of f' may show a transition to another curve of the plotted f' data, in other words a break in its slope. Here, we applied the method to characterize the effects of toxins on the flexibility of actin filaments.

2.5. Differential scanning calorimetry

The thermal denaturation of Ca-actin filaments was monitored between 0 and 100 °C with a SETARAM Micro DSC-II calorimeter. The heating and cooling rates were 0.3 K/min. Conventional Hastelloy batch vessels were used with 850 μl sample volume and the actin concentration was 3 mg/ml. Buffer A was used as reference. The sample and reference vessels were balanced with a precision of ±0.1 mg. Calorimetric enthalpy change (ΔH) of endothermic transitions was calculated from the area under the heat absorption curve using two-point setting SETARAM peak integration. Transition entropy change (ΔS) was calculated for the peak transition temperature (T_m) from the following equation:

$$\Delta S = \Delta H/T_m \quad (4)$$

The Gibbs free enthalpy change was calculated from the following equation:

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

3. Results and discussion

In this work our aim was to compare the effect of phalloidin and jasplakinolide on the dynamics of actin filaments. The dynamic behavior of the filaments was sensitive to whether they were polymerized in the presence of calcium (Ca–F-actin) or magnesium ions (Mg–F-actin) [12–14]. In previous FRET experiments the intermonomer flexibility of Ca–F-actin was greater than that of Mg–F-actin [12]. The filament flexibility showed little dependence on the presence of phalloidin in the case of Mg–F-actin (inset in Fig. 2a, and [15]), while for Ca–F-actin the filament flexibility was greatly reduced after phalloidin binding. Therefore, we have chosen Ca–F-actin as a model system in this study.

Jasplakinolide and phalloidin bind actin filaments tightly with affinities of 15 and 36 nM, respectively [2,3], suggesting that under the conditions applied here (20 μM actin and toxin) more than 99% of the actin protomers bound toxin in the filaments.

3.1. FRET experiments

We used FRET methods to describe the effects of phalloidin and jasplakinolide on the flexibility of actin filaments. In one set of experiments, the separately labeled IAEDANS-actin and IAF-actin monomers were mixed before polymerization. These experiments therefore reported on the intermonomer flexibility of actin filaments. In another set of experiments the double labeled IAEDANS–FC-actin monomers were incorporated into actin filaments, and the intramonomer flexibility of individual actin protomers within the filaments was characterized. The interpretation of the temperature dependent FRET experiments assumes that no major conformational changes occur [11]. It was previously shown that in the absence of toxins this condition was fulfilled [13]. In the present work the temperature profile of relative f' was smooth in the presence of toxins as well, which suggests that the temperature did not induce major conformational changes in the actin filaments. At the same time, the calculated apparent donor–acceptor distances were temperature independent in the presence of phalloidin or jasplakinolide (data are not shown), i.e., only the relative fluctuation of the probes was affected by the temperature.

In intermonomer FRET experiments the fluorescence intensity of IAEDANS (donor) was smaller in the presence of IAF (acceptor) than in the absence of it between 400 and 460 nm due to the effective quenching by the acceptor (Fig. 1). The FRET efficiency was calculated (Eq. (2)) and the f' was determined (Eq. (3)) as a function of temperature between 6 and 34 °C. The relative f' was determined by normalizing the f' by its value at 6 °C (Fig. 2a). For Ca–F-actin the relative f' increased by 135% in the absence of toxins. In the presence of phalloidin the temperature induced change of the relative f' was 13%, while only 2% increase was observed in the presence of jasplakinolide (Fig. 2a). The smaller temperature induced increase of f' in the presence of toxins indicated that the monomer–monomer interactions along the actin filament became stiffer and the flexibility of the actin filaments decreased after the binding of either phalloidin or jasplakinolide. The effect of jasplakinolide was slightly greater than that of the phalloidin.

The dynamic properties of actin filaments can be altered by the modification of the intramolecular monomer flexibility as

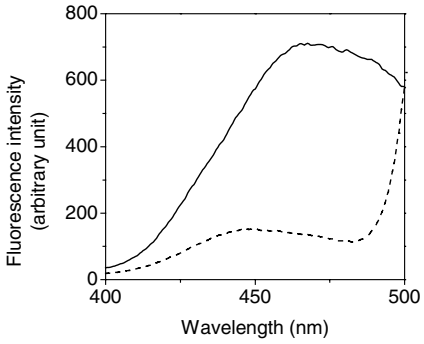

Fig. 1. Fluorescence emission spectra of IAEDANS labeled actin filament in the absence (solid line) and in the presence of IAF monomers (dashed line). The excitation wavelength for IAEDANS was 350 nm and the emission was monitored between 400 and 500 nm. The optical slits were set to 5 nm in both the excitation and emission sides. The spectra were recorded at 22 °C in buffer A.

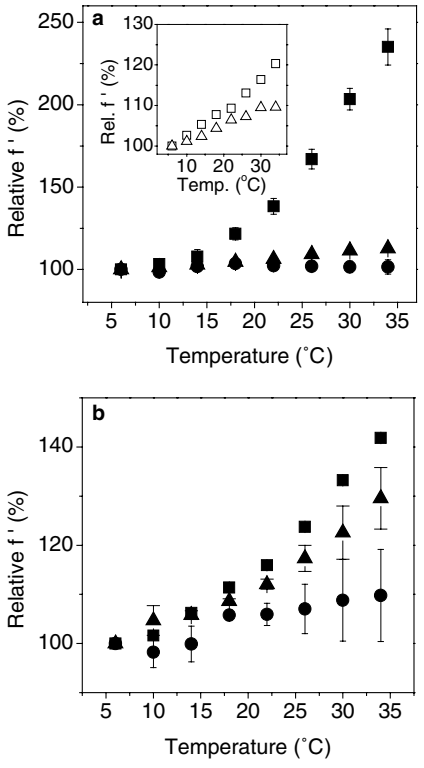

Fig. 2. Temperature dependence of the relative f' of Ca–F-actin calculated from the intermonomer (a) and intramonomer (b) FRET data. The data obtained in the absence of toxins are presented as filled squares. The figure also shows the temperature profile of the relative f' in the presence of phalloidin (filled triangles), or jasplakinolide (filled circles). The inset in panel *a* shows the temperature dependence of the relative f' for Mg–F-actin in the absence (empty squares) and presence (empty triangles) of phalloidin calculated from the intermonomer FRET data. The errors presented are standard errors. The experimental conditions were as in Fig. 1, except that the temperature was adjusted as shown.

well. In intramonomer FRET experiments carried out with the IAEDANS(Cys^{374})–FC(Gln^{41}) pair, the distances calculated from the measured FRET efficiencies ranged between 4.6 and 4.9 nm. These values are in agreement with the distance obtained between the Gln^{41} and Arg^{372} residues from the atomic mode of the actin monomer (4.7 nm, [16]), which suggests that the effect of intermonomer FRET was negligible. The relative f' increased by 42% in the absence of toxins (Fig. 2b). In the presence of phalloidin the relative f' increased by 30%, while the change was only 10% with jasplakinolide. These data indicated that the flexibility of the actin protomers decreased after the binding of toxins. In correlation with the intermonomer FRET data, the intramonomer FRET results showed that the binding of jasplakinolide induced greater dynamic changes than phalloidin in the actin filaments.

3.2. Differential scanning calorimetry

The calorimetric curves were obtained between 0 and 100 °C for Ca–F-actin in the absence or presence of toxins (Fig. 3). The repeated scans measured after the samples were cooled showed that the phase transition processes were irreversible (data are not shown). The results showed small but reproducible transition at lower temperatures (between ~40 and 50 °C) (Fig. 3). The calorimetric enthalpy attributed to this transition increased in the presence of phalloidin, while jasplakinolide decreased the enthalpy substantially. Considering that in previous studies solution of actin monomers gave T_m value of 53.4 °C [17–19], one may speculate that the actin monomer content of the samples was responsible for the observations. However, in the case of actin concentrations applied here (46 μM) only less than 0.5% of the actin should have been in the monomeric form considering the critical concentration for monomer assembly (~0.1 μM), and thus this monomer content appears to be much less than enough to account for the low temperature transitions. On the other hand, in the presence of phalloidin the monomer concentration should decrease, thus the observed phalloidin effect on the low temperature transition (Fig. 3) was not consistent with the hypothesis that actin monomer denaturation was observed between 40 and 50 °C. It seems that based on the present data set the proper identification of the low temperature transitions is difficult, and further experiments are required to clarify the origin of these low temperature heat effects.

There was a larger transition at higher temperatures (>60 °C) (Fig. 3), which we attribute to the thermal denaturation of the filaments. The melting temperature (T_m) from this transition was 67.3 °C in the absence of toxins, and shifted to 79.3 and 87.7 °C in the presence of phalloidin or jasplakinolide, respectively (Table 1). The effect of phalloidin was similar to

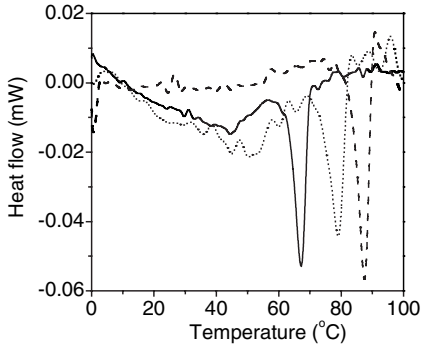

Fig. 3. The denaturation curves for Ca–F-actin in the absence of toxins (solid line) and in the presence of phalloidin (dotted line) or jasplakinolide (dashed line).

Table 1
The thermodynamic parameters obtained for Ca–F-actin from differential calorimetric experiments in the absence and the presence of phalloidin or jasplakinolide

Toxin	T_m (°C)	ΔH (J/mol)	ΔS (J/mol K)	ΔG (J/mol)
None	67.3 ± 0.5	720 ± 41	2.2 ± 0.1	128 ± 7
Phalloidin	79.3 ± 0.6	936 ± 55	2.7 ± 0.2	210 ± 10
Jasplakinolide	87.7 ± 0.7	1117 ± 64	3.1 ± 0.2	271 ± 15

T_m is the peak temperature of endothermic melting, ΔH is the calorimetric enthalpy change of the high temperature transition, ΔS is the entropy change at T_m and ΔG is the Gibbs free enthalpy calculated for room temperature. The errors presented are standard deviations.

that observed previously [20,21]. The larger T_m is attributed to the greater resistance to heat denaturation, and thus the variation of the T_m values indicated that the thermal stability of Ca–F-actin was increased after the binding of either phalloidin or jasplakinolide. In correlation with these results the calorimetric enthalpy, entropy and Gibbs free enthalpy changes were greater in the presence of toxins (Table 1), indicating that larger thermal influence was required in these cases to denature the actin filaments. These observations suggest that the filament structure was stabilized by phalloidin or jasplakinolide. In agreement with the fluorescence data, the stabilizing effect of jasplakinolide was greater on actin filaments than that of phalloidin.

Despite the many advantages of the application of phalloidin, an important disadvantage came from the fact that cell membranes are not permeable for this toxin, and thus the cell membrane must be artificially permeabilized for intracellular investigations. The cell membrane is permeable for jasplakinolide. Our biophysical characterization of the effects of jasplakinolide on the conformational and dynamic properties of actin filaments can help to estimate the limits and possibilities of in vivo assays with jasplakinolide, and to design and apply new, e.g., fluorescent, jasplakinolide derivatives.

Acknowledgements: This work was supported by grants from the National Research Foundation (OTKA grant Nos. T32700, T34442, T43103), by a grant of Ministry of Education (615/2003), by the Hungarian Academy of Sciences and NKFP 1/026/2001. The SETARAM Micro DSC-II was payed for from the OTKA CO-272 grant. Miklós Nyitrai is an EMBO/HHMI Scientist. Gábor Hild is financially supported by the János Bolyai Research Fellowship of the Hungarian Academy of Sciences.

References

[1] Miyamoto, Y., Kuroda, M., Munekata, E. and Masaki, T. (1986) J. Biochem. (Tokyo) 100, 1677–1680.
[2] Faulstich, H., Schafer, A.J. and Weckauf, M. (1977) Hoppe Seylers Z. Physiol. Chem. 358, 181–184.
[3] Bubb, M.R., Senderowicz, A.M., Sausville, E.A., Duncan, K.L. and Korn, E.D. (1994) J. Biol. Chem. 269, 14869–14871.
[4] Bubb, M.R., Spector, I., Beyer, B.B. and Fosen, K.M. (2000) J. Biol. Chem. 275, 5163–5170.
[5] Spector, I., Braet, F., Shochet, N.R. and Bubb, M.R. (1999) Microscopy Research and Technique 47, 18–37.
[6] Feuer, G., Molnár, F., Pettkó, E. and Straub, F.B. (1948) Hung. Acta Physiol. 1, 150–163.
[7] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246 (15), 4866–4871.
[8] Miki, M., dos Remedios, C.G. and Barden, J.A. (1987) Eur. J. Biochem. 168, 339–345.
[9] Hild, G., Nyitrai, M. and Somogyi, B. (2002) Eur. J. Biochem. 269, 842–849.
[10] Takashi, R. (1988) Biochemistry 27, 938–943.
[11] Somogyi, B., Matko, J., Papp, S., Hevessy, J., Welch, G.R. and Damjanovich, S. (1984) Biochemistry 23, 3403–3411.
[12] Hild, G., Nyitrai, M., Belagyi, J. and Somogyi, B. (1998) Biophys. J. 75, 3015–3022.
[13] Nyitrai, M., Hild, G., Belagyi, J. and Somogyi, B. (1999) J. Biol. Chem. 274, 12996–13001.
[14] Orlova, A. and Egelman, E.H. (1993) J. Mol. Biol. 232, 334–341.
[15] Nyitrai, M., Hild, G., Hartvig, N., Belagyi, J. and Somogyi, B. (2000) J. Biol. Chem. 275, 41143–41149.
[16] Kabsch, W., Mannherz, H., Suck, D., Pai, E. and Holmes, K. (1990) Nature 347, 37–44.
[17] Bertazzon, A., Tian, G.H., Lamblin, A. and Tsong, T.Y. (1990) Biochemistry 29, 291–298.
[18] Lőrinczy, D. and Belágyi, J. (1995) Thermochim. Acta 259, 153–164.
[19] Lőrinczy, D., Könczöl, F., Gaszner, B. and Belágyi, J. (1998) Thermochim. Acta 322, 95–100.
[20] Le Bihan, T. and Gicquaud, C. (1991) Biochem. Biophys. Res. Commun. 181, 542–547.
[21] Levitsky, D.I., Rostkova, E.V., Orlov, V.N., Nikolaeva, O.P., Moiseeva, L.N., Teplova, M.V. and Gusev, N.B. (2000) Eur. J. Biochem. 267, 1869–1877.